Derivatization of Glass and Polypropylene Surfaces

This unit describes the derivatization of solid support media with a versatile linker system. The procedure permits the production of various linker types whose characteristics can be tailored to the requirements of the eventual application. The protocols described here have been used extensively on both nonporous (glass, polypropylene foil) and porous (nylon or polypropylene membrane) substrates. If required, a dendrimeric structure can be formed, thereby increasing the loading capacity of the surface in a stepwise fashion, which is especially useful for glass surfaces.

A prerequisite for derivatization is the presence of anchoring groups (hydroxyl or amino groups) on the support surface. These anchoring groups may be intrinsic to the medium (e.g., amino functions on nylon) or may be introduced by chemical modification. Standard modifications include silanization reactions on glass (see Support Protocol 1) or plasma-amination on polypropylene. The latter process is a standard surface modification technique in the plastics industry. The authors obtained plasma-aminated material from AIMS Scientific Products, which processed polypropylene foils provided by the authors.

Generally, the linker synthesis described below (see Basic Protocol) consists of a two-step process (Fig. 12.4.1) that may be repeated in an iterative way. In a first step, the anchoring groups on the surface are activated by an acylation reagent (e.g., acryloyl chloride, 4-nitrophenyl chloroformate). Subsequently, in a second step, these activated intermediates are reacted with an amine component. Preferably, a polyamine is employed, since this increases the surface loading. By careful selection of the polyamine component, the surface properties (e.g., linker length, hydrophobicity, charge) may be customized and adapted to specific requirements. The performance of the derivatization process can be

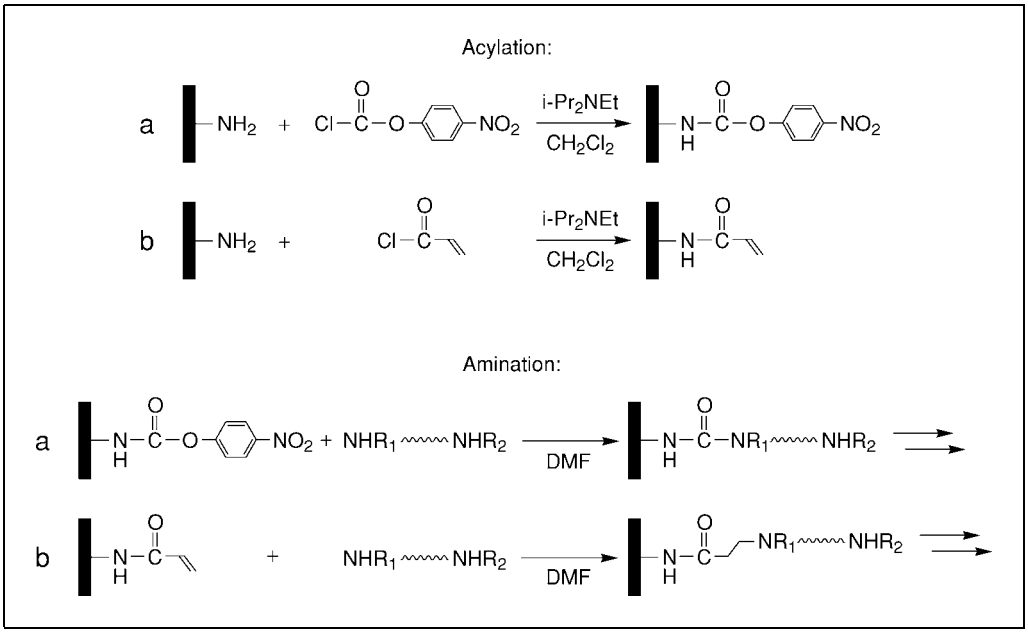

Figure 12.4.1 Linker synthesis on an aminated support. A two-step process of acylation (activation; top) and reaction with various amines (bottom) is used to derivatize the solid support with an appropriate linker. The process is depicted using activation with (**a**) 4-nitrophenyl chloroformate and (**b**) acryloyl chloride.

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monitored by analyzing the density of amino functions on the surface via bromphenol blue staining (see Support Protocol 2).

For the immobilization of existing nucleic acids, an additional activation step is required to generate a covalent linkage between biomolecule and linker. For that purpose, bifunctional cross-linking reagents are used (see Support Protocol 3). In situ synthesis of DNA microarrays does not require such activation but can proceed directly, since the terminal groups of the linker serve as starting points for oligonucleotide synthesis.

NOTE: Use deionized, distilled water in all recipes and protocol steps. Use dry reagent-grade solvents for all reactions.

BASIC PROTOCOL

DERIVATIZATION OF SOLID SUPPORT

The process described below permits the modification of glass and polypropylene surfaces forming a linker of dendrimeric structure. The derivatization is used either for attaching prefabricated DNA-oligonucleotides, PCR products, and peptide nucleic acid (PNA) oligomers, or for the in situ synthesis of DNA microarrays (*UNIT 12.3*).

Materials

Acetone
Nitrogen
Diisopropylethylamine (DIPEA)
Acetonitrile, anhydrous
4-Nitrophenyl chloroformate or acryloyl chloride
Dichloroethane, dry
Amine compound—e.g., tetraethylenepentamine; 1,4-*bis*-(3-aminopropoxy)butane; 4-aminomethyl-1,8,-octadiamine; 4,7,10-trioxa-1,13-tridecandiamine; *N*,*N*-dimethyl-1,6-hexadiamine; 2-(2-aminoethoxy)ethanol; jeffamine; 3-amino-1,2-propandiol
Dimethylformamide (DMF), dry and amine free
Methanol, reagent grade
18 × 8–cm polypropylene vessels with tight-fitting lids
Solid support medium (e.g., glass, polypropylene foil, nylon membrane, polypropylene membrane) with anchoring groups in place on surface (e.g., see Support Protocol 1 for silanizing glass)
Orbital shaker

Activate solid support

1. Clean an 18 × 8–cm polypropylene vessel carefully with acetone and dry thoroughly.

   Any polypropylene vessel can be used if it is of sufficient size to act as a reaction container for the derivatization of the support medium in bulk. It must also have a lid that fits tightly to prevent moisture from entering the reaction chamber. An 18 × 8–cm container can be used to process up to six microscope slides in parallel or one ~17 × 7.5–cm sheet of polypropylene foil, and will have a wash volume of ~30 to 40 ml.

2. Flush the container with nitrogen and fill with a solution of 171 µl (1 mmol) DIPEA in 30 mL anhydrous acetonitrile.

3. Add one of the following:

   192 mg (1 mmol) 4-nitrophenyl chloroformate
   81 µl (1 mmol) acryloyl chloride.

   See Background Information for a discussion of these activating agents.

4. Add the solid support medium (with anchoring groups) and close the lid carefully to seal the reaction chamber.
5. Incubate 2 hr at ambient temperature on an orbital shaker.
6. Remove the reaction solution, wash the supports thoroughly (two times) with 30 to 40 ml dichloroethane, and then dry under a flush of nitrogen.

   It is not advisable to store the activated supports. It is best to proceed directly to the reaction with the amine component.

React with amine

7. Clean a second polypropylene container carefully with acetone and dry thoroughly.
8. Flush the container with nitrogen and fill it with a solution of 1 mmol of the desired amine component in 30 mL anhydrous amine-free DMF.

   In principle, any amine reagent can be employed in this reaction (see Commentary and materials section for suggestions).

9. Add the activated solid support medium to the amine reaction solution and close the lid carefully to seal the reaction chamber.
10. Incubate at ambient temperature on an orbital shaker for 12 hr if 4-nitrophenyl chloroformate was used for activation or 24 hr if acryloyl chloride was used.

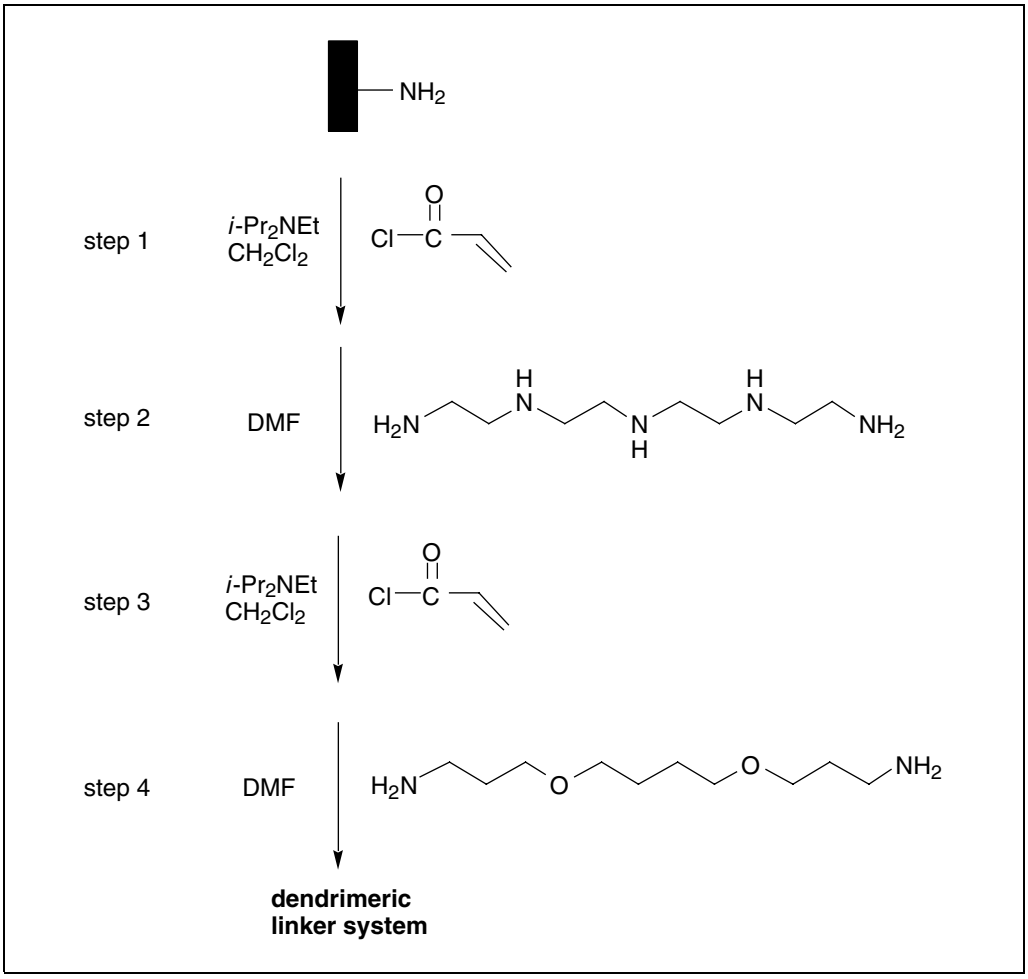

Figure 12.4.2 By an iterative process of activation and amination, linker structures of different complexity and diverse inherent characteristics can be synthesized.

The nucleophilic substitution of the 4-nitrophenyl moiety by an amine produces a colored group, which allows monitoring of the reaction by visual inspection.

11. Remove the reaction solution and wash the support media thoroughly with DMF, then methanol, and finally acetone. Dry under a flush of nitrogen.

12. If desired, perform another round of activation and amine reaction in order to increase surface loading/dendrimeric character of the support or to alter the surface properties.

    For the derivatization of microarray glass surfaces used for the immobilization of nucleic acids or in situ synthesis of DNA oligonucleotides, the best results are obtained with two reaction cycles consisting of acryloyl chloride (activation), tetraethylenepentamine (amine derivatization), acryloyl chloride (activation), and 1,4-bis-(aminopropoxy)butane (amine derivatization). This is illustrated in Figure 12.4.2.

13. Store derivatized support in a dry place up to several weeks at 4°C

SUPPORT PROTOCOL 1

SILANIZATION OF GLASS SLIDES

Prior to the actual silanization, the glass is etched, thereby improving the overall quality of the process. Both processes are described below.

Materials

10% (w/v) aqueous NaOH
1% (v/v) aqueous HCl
Methanol, reagent grade
3% (w/v) aminopropyltrimethoxysilane in 95% (v/v) methanol
Nitrogen

Glass microscope slides (e.g., Menzel-Gläser, Germany)
Several glass vessels with lids (e.g., hematology staining jar)
Orbital shaker
Polypropylene vessel with lid
Bath sonicator
110°C oven

Etch glass surface

1. Immerse underivatized glass microscope slides in a glass vessel filled with a 10% (w/v) NaOH solution and close the lid.

   Etching can be performed in the type of glass staining jar frequently used in hematology for labeling experiments. These allow eight to ten slides to be placed standing within the vessel.

2. Shake gently overnight on an orbital shaker.

3. Remove slides from the NaOH bath using tweezers.

4. Wash by immersing the slides in a glass vessel filled with water and shaking briefly (2 to 3 min) on an orbital shaker.

5. Wash in another glass vessel, filled with 1% (v/v) HCl, by shaking 2 to 3 min on an orbital shaker.

6. Wash 2 to 3 min in water.

7. Immerse the slides in a glass vessel filled with methanol and shake 2 to 3 min on an orbital shaker. Proceed directly to the silanization reaction.

Silanize etched surface

8. Fill a polypropylene container with 3% (w/v) aminopropyltrimethoxysilane in 95% (v/v) methanol.
9. Immerse etched glass slides in the container so that they just cover the bottom in a single layer. Close the lid.

   A single layer is used to prevent adherence between the slides.
10. Transfer the container to a bath sonicator. Sonicate 15 min at ambient temperature.

    CAUTION: *The reaction mixture becomes hot during sonication. Open lid carefully.*
11. Remove slides with tweezers.
12. Wash by immersing the slides in a glass vessel filled with methanol and shaking 2 to 3 min on an orbital shaker. Use very gentle agitation to prevent the slides from moving and adhering to one another.
13. Wash 2 to 3 min in water on an orbital shaker.
14. Dry slides under a stream of nitrogen gas.
15. Transfer slides to a preheated 110°C oven for 15 min.
16. Remove slides from oven and store up to several weeks at a convenient temperature.

QUALITY CONTROL OF DERIVATION REACTIONS

SUPPORT PROTOCOL 2

To control the efficiency of the derivatization procedure, a bromphenol blue staining reaction is employed. The quality is assessed by measuring the amount of surface-bound amino functions and hence the surface loading. This assay is preferably carried out on a control strip made of the appropriate support material and added to the derivatization reactions. After each amination step, a part of the control strip is removed and analyzed.

For glass supports, only qualitative assessments can be made because of the low loading capacity of glass. Because the blue color achieved with glass can barely be visualized by eye, a polypropylene control strip is used in the same reactions with the glass slides. If a strong loading (blue color) is achieved on the polypropylene control strip, it can be assumed that the derivatization reaction in general was successful and that the slides are usable.

Quantitative assessments can be made with polypropylene foils and membranes, and with nylon membranes, all of which have sufficiently high loading to obtain reliable readings by spectrophotometry. For quantitative purposes, the control strip must be of the identical support medium used for derivatization.

Additional Materials *(also see Basic Protocol)*

Control strip: hydroxylated or aminated solid support (see Basic Protocol 1)
Amine-free DMF containing 0.05% bromphenol blue
Ethanol, reagent grade
20% piperidine in DMF
Spectrophotometer, 605 nm

1. Add a control strip to the reactions described in the Basic Protocol. For quantification, use the identical support material that was derivatized for use.
2. At each step in the derivatization, remove an ~1-cm^2 portion of the control strip.

   For quantification and comparison purposes, always take identical portions.

3. Transfer to a new vessel and wash four times with 10 to 15 mL amine-free DMF.
4. Transfer to 2 mL amine-free DMF containing 0.05% (w/v) bromphenol blue and incubate 5 to 10 min with gentle agitation.

   In the presence of amino functions on the support surface, the support will turn blue and the staining solution will turn yellow.

5. Transfer to a new vessel and wash three times with ethanol to remove any residual stain.
6. Transfer to 20% piperidine in amine-free DMF to destain. Agitate gently until the blue color of the control strip is completely removed.
7. Collect the destain solution and analyze in a spectrophotometer at 605 nm (ε_{605} = 95,000 M^{-1} cm^{-1}). If necessary, dilute solution with 20% piperidine in DMF.
8. Compare the blue color/absorption of different rounds of derivatization to monitor surface loading.

   For polypropylene, the actual number of amino functions per area can be calculated. For glass, only relative measurements across reactions are possible.

SUPPORT PROTOCOL 3

IMMOBILIZATION OF 5′-AMINO-LABELED NUCLEIC ACID COMPOUNDS

While in situ DNA synthesis can proceed directly on the linker resulting from two subsequent reaction cycles with acryloyl chloride, tetraethylenepentamine, acryloyl chloride, and 1,4-bis-(aminopropoxy)butane (see Basic Protocol), an additional activation step is required for the covalent attachment of prefabricated 5′-amino-labeled nucleic acid compounds (oligonucleotides, PCR products, peptide nucleic acids).

Additional Materials *(also see Basic Protocol)*

Phenylene diisothiocyanate (PDITC) *or* dimethylsuberimidate dihydrochloride (DMS)
10% (v/v) anhydrous pyridine in amine-free DMF (for PDITC)
Saturated aqueous sodium bicarbonate ($NaHCO_3$; for DMS)
5′-Amino-labeled nucleic acid
Diisopropylethylamine (DIPEA)
1 mM Tris·Cl, pH 7.5 (see *APPENDIX 2A*; optional)
6-Amino-1-hexanol

Amino-functionalized glass slides or polypropylene sheets (see Basic Protocol)
37°C humid chamber

Activate linker

For activation with PDITC

1a. Fill an 18 × 8–cm polypropylene container with 192 mg (1 mmol) PDITC in 40 mL of 10% (v/v) anhydrous pyridine in DMF.

2a. Immerse amino-functionalized glass slides or polypropylene sheets in activation solution and incubate for 2 hr with gentle agitation on an orbital shaker.

3a. Wash twice with 30 to 40 mL amine-free DMF, shaking gently for 2 to 3 min each.

4a. Wash twice as above with dichloroethane.

5a. Dry slides under a stream of nitrogen gas.

For activation with DMS

1b. Fill an 18 × 8–cm polypropylene container with 273 mg (1 mmol) DMS in 40 mL saturated $NaHCO_3$.

2b. Immerse the amino-functionalized glass slides or polypropylene sheets in activation solution and incubate for 1 hr with gentle agitation on an orbital shaker.

3b. Wash twice with 30 to 40 mL water, shaking gently for 2 to 3 min each.

4b. Wash twice as above with acetone.

5b. Dry slides under a stream of nitrogen gas.

Spot nucleic acids onto activated surfaces

6. Take up a 5′-amino-labeled nucleic acid in 1% (w/v) DIPEA in water or 1 mM Tris·Cl, pH 7.5.

7. Administer droplets of 0.1 to 50 nl onto the activated support.

8. Incubate in a humid chamber at 37°C overnight.

9. Wash twice with 30 to 40 mL water, shaking gently for 2 to 3 min each.

10. Wash twice as above with methanol.

11. Deactivate by incubating 2 hr in a freshly made solution of 50 mM 6-amino-1-hexanol and 150 mM DIPEA in DMF.

12. Wash the DNA arrays successively (two times each) with DMF, acetone, and water.

13. Store arrays dry for 4 to 8 weeks at 4°C.

COMMENTARY

Background Information

This unit describes the synthesis of a flexible linker system on glass and polypropylene support media (Beier and Hoheisel, 1999). The reaction scheme consists of a two-step procedure: an initial activation followed by a reaction with an amine, preferentially a polyamine. In the first step, a surface-bound anchoring group is reacted with 4-nitrophenyl chloroformate to form an activated ester, or with acryloyl chloride to form an α,β-unsaturated carbonyl derivative (Fig. 12.4.1). Both products represent activated species that are reactive towards an amine derivative. The reaction of the nitrophenyl ester with an amine proceeds quickly, whereas the α,β-unsaturated carbonyl derivative needs a prolonged 24-hr reaction time. However, because the latter leads to better final hybridization results, activation with acryloyl chloride is preferred.

After having completed a first cycle of derivatization (activation plus amine coupling), the very same procedure may be repeated with the same or a different amine reagent. By simple variation of the amino components, different linker types can be synthesized. The linker properties can be modulated in many ways, from merely increasing the distance between the support surface and the biomolecule to be attached, to multiplying the loading capacity. If a polyamine such as tetraethylenepentamine is utilized, for example, the loading capacity of the support should be increased approximately five-fold. Since polyamines contain both primary and secondary amino functions, one unique product is not generated, but rather a mixture of compounds. Furthermore, the number of amino functions incorporated during linker synthesis controls the number of positive charges at neutral pH. Employing the amines found in the materials section of the Basic Protocol, a large number of different linker types have been synthesized by this reaction scheme (Beier and Hoheisel, 1999). The support characteristics can be tailored to the requirements of the intended applications.

Many linker systems were found to be well suited for binding nucleic acids; however, one system in particular proved to be excellent under all conditions and was used for attaching prefabricated DNA oligonucleotides, PCR products, and peptide nucleic acid (PNA) oligomers, as well as for in situ synthesis of

DNA microarrays. It is generated preferably on aminated (glass) surfaces by sequential reactions with acryloyl chloride, tetraethylenepentamine, acryloyl chloride, and 1,4-bis-(aminopropoxy)butane (Fig. 12.4.2). For in situ synthesis of DNA microarrays (Pease et al., 1997; Beier and Hoheisel, 2000), no further derivatization step is required. The terminal amino group of the linker serves directly as the starting point for oligonucleotide synthesis.

For immobilization of prefabricated nucleic acids, an additional activation is needed to generate a covalent linkage between biomolecule and support. For that purpose, bifunctional cross-linking reagents like phenylene diisothiocyanate or dimethylsuberimidate are utilized (Beier and Hoheisel, 1999). Activating the solid-phase surface prevents any cross-reaction between the nucleic acids prior to immobilization. Prior to spotting onto the support, a base is added to the nucleic acid solution, since the cross-linking agents have their optimal reactivity in a basic milieu. Because of its non-nucleophilic character, diisopropylethylamine is employed, since it does not compete for the reactive sites on the support. However, immobilization also works at neutral pH (i.e., in water). Since small droplets evaporate quickly after application, the reaction is run to completion by incubating the DNA arrays in a humid chamber at 37°C after administration of the spots. Alternatively, betaine may be used to reduce evaporation (Diehl et al., 2001).

Critical Parameters

Overall, the procedure is very robust. The most critical parameter in actual routine processing is preventing moisture from entering the reaction chamber during the two-step derivatization. Also, there should be no lengthy time interval between activation and amination.

Anticipated Results

Building a dendrimeric structure increases the loading capacity significantly, which can be a critical factor for glass supports in particular. With the protocols for coupling nucleic acids as detailed above, an increase by ten-fold has been observed.

Time Considerations

The overall preparation time depends on the number of iterative activation and amination steps performed and is mainly determined by the incubations times. About 1 day should be considered for each cycle of activation and amination. However, even if several iterative cycles are performed, throughput is no major limitation, since surface modification can be performed in bulk.

Literature Cited

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